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ABSTRACT

Dangke is the Indonesian cheese produced from bovine milk using latex from young papaya to coagulate casein. Dangke is generally consumed by Indonesian people located in South Sulawesi Province. In line with demand of Dangke, the preservation is needed. Since, there was no literature which was found about preservation of Dangke, this study is aimed at knowing the quality of Dangke based on total bacteria and pH value stored in antimicrobial agent of lactoferrin and lactoperoxidase system from bovine whey, aquadest and phospate buffer at ambient temperature for 12 h. The lactoferrin, lactoperoxidase and whey were obtained from bovine milk and purified using ion exchange chromatography method. The result of the study showed that lactoperoxidase system provide remarkable effect of decreasing total bacteria from 8 log CFU mL^{-1} to 5 log CFU mL^{-1} while other storage solutions have no antimicrobial activity against bacteria in Dangke. The pH value of Dangke was stable when stored in lactoferrin and lactoperoxidase system. Since, both of these preservatives could be categorized as safe, the application in Dangke may open the alternative method to store Dangke.

Key words: Dangke, lactoferrin, lactoperoxidase, whey, total bacteria

INTRODUCTION

Dangke is a traditional cheese from South Sulawesi Province in Indonesia. Dangke is mostly made from cow's milk but buffalo's milk or their mixture can also be used. Dangke is a semi solid and salty cheese that available in the traditional market and traditionally manufactured by local people. A small amount of papain has been used to coagulate casein from whey. After whey removal, the mild pressure is usually applied to produce semi solid cheese. The compositions of Dangke are 47.75% of water, 2.32% of ash, 33.89% of fat and 17.01% protein (Marzoeki *et al*., 1978). The process of making Dangke initially is started by heating in low temperature for long time (65°C, 30 min) and for casein coagulation, subsequently 5 g of papain is added into milk. The addition of papain exerts bitter taste since the papain may promote the hydrophobic groups generation (Amri and Mamboya, 2012). The bitterness taste of Dangke may be neutralized by the addition of salt. It has been understood that salt may also inhibit the spoilage of bacteria (Beresford *et al*., 2001). Native people commonly consume Dangke for the complimentary of their food, so the salt may promote the better taste in food (Sirajuddin *et al*., 2013).

Dangke manufacturing is mostly made from cow's milk but sheep's and goat's milk or a mixture of them. Since, the local people consume Dangke daily, they did not pay high attention for the preservation because local people will consume it immediately after manufacturing. However, since the number of local people is travelling from and to this province, the demand has increase resulting in the need for preservation. Natively, Dangke's shelf live is relatively short (about six hours), this is because Dangke is made from fresh milk that contains various elements and mostly consists of food substance that is also needed for bacteria growth. One of methods to extend the storage period of food product is the preservation by using antimicrobial substances or compounds.

The preservative for prolonging the shelf live of Dangke may be obtained from chemicals however, since the people may pay much more concern on their health, the chemicals based preservation may be avoided. In line with this demand, researchers pay much more attention for the utilization of the Generally Recognize As Safe (GRAS)'s preservatives. Lactoferrin or most commonly called lactotransferrin is transferrin that is isolated from milk. Lactoferrin is antimicrobial agent because it contains glycoprotein-703 amino acid that has extremely high ability to bind Fe from microbe, so that it significantly inhibits microbe growth (Conneely, 2001). Lactoperoxidase system is widely known as a system that naturally exists in fresh milk as antimicrobial. Lactoperoxidase system has been proven for being active to positive and negative gram microorganism (Naidu, 2000; Marks *et al*., 2001). Lactoperoxidase system catalyses reaction of hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-) that occur naturally in milk to become a compound named hypothiocyanite ($OSCN^-$) (Barrett *et al*., 1999; Kussendrager and van Hooijdonk, 2000; Seifu *et al*., 2007). The $OSCN^-$ is a compound that takes responsibility for killing bacteria, fungi and virus by breaking down sulfhydryls groups (S-H group) from cell membrane causing vital impairment of cell membrane finally leading to the death of the cell (Al-Baarri *et al*., 2011a; Borch *et al*., 1989; Dajanta *et al*., 2008; Touch *et al*., 2004).

Based on the remarkable antimicrobial activity of lactoferrin and lactoperoxidase system and there is no study that was found in the preservation of both compound in Dangke, this study was aimed at analysis of total bacterial growth and pH value of Dangke stored at ambient temperature. The result of this study may provide an alternative way for Dangke's storage.

MATERIAL AND METHODS

Materials: Fresh bovine milk was provided by Campus Farm in Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang-Indonesia. Papain enzyme was obtained from 3-4 month old fresh papaya fruits. Commercial microbial rennet was obtained from Singapore. The spectrophotometer (Mini UV-1800, Schimadzu, Japan) was used for analysis of LPO activity and detection of protein concentration. The H_2O_2, KSCN, 2, 2-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS) were purchased from Sigma. Unless other specified compound were reagent grade.

Whey preparation: The whey was prepared as method conducted by Al-Baarri *et al*. (2011b) without any modification.

LPO production from whey: Whey was used for production of lactoperoxidase and lactoferrin through ion exchange method using SP Sepharose Fast Flow Column (GE Healthcare Bio-Science AB, Sweeden, Lot. No. 10081054). Subsequently, 0.4 M NaCl in 300 mL of 0.1 M PB (pH 7.0) was flowed into SP Sepharose® Fast Flow in order to generate lactoperoxidase solution. Three hundred millilitres of 1 M NaCl in 0.1 M PB (pH 7.0) was then poured to produce lactoferrin solution. Each

eluate obtained from above mentioned method was analyzed for approximate protein concentration in each tubes (10 mL $tube^{-1}$) using spectrophotometer and its absorbance was measured at 280 nm. Top ten highest absorbance of tubes after peak were collected to determine the LPO enzyme activity using ABTS at 412 nm (Al-Baarri *et al*., 2011b). To check the purity of lactoperoxidase and lactoferrin, the SDS PAGE was applied.

Manufacture of Dangke: Procedure of Dangke's making was adapted from method of JICA (2009). It was started by a heating of 3 L of fresh bovine milk at 60°C for 30 min. The next step was the addition of 0.03% (v/v) papain enzyme. After aglutination occurred, the whey was drained by using sterile filter cloth. The curd was then stored in ambient temperature and gently pressed for 3 h to produce the Dangke.

Microbial count: Petrifilm Aerobic Count Plates (3 M Microbiology, St. Paul, Minn., U.S.A.) was used to count the microbial appeared in Dangke. After manufacture, Dangke was cut into cube with the approx. of weight 1 g. The number of total bacteria in Dangke in the presence of lactoperoxidase system was determined as follows: 1 g Dangke was stored at 1000 μL hyphothiocyanite-rich-solution and incubated for 6 h at 30°C. Hyphothiocyanate-rich-solution was made from the addition of 250 μL of 1.0 mM H_2O_2 and 250 μL of 1.0 mM KSCN into 500 μL of LPO solution (35 U mL^{-1}). After incubation at 30°C for 10 min, hyphothiocyanite-rich-solution should be generated. Enumeration of bacteria was done by counting the solution that was obtained from serial dilutions of the assay mixture with a sterile 0.88% NaCl solution. The diluted mixture (1000 μL) was spread onto plates. The plate were incubated at 37°C for 48 h. The CFU of microbes in the sample solution were counted on the plates.

Statistical analysis: Total bacteria of Dangke stored in various storage solutions for 12 h were analyzed statistically using one-way analysis of variance (ANOVA) and the means were compared by the Duncan test at a significant level of 0.05 (Free Statistical Software Package R for Macintosh, U.S.A).

RESULT AND DISCUSSION

Purification of lactoperoxide and lactoferrin: Lactoperoxidase and lactoferrin was obtained from whey using ion exchange chromatography method. Both components were collected from top ten highest absorbance of tubes after peak at 280 nm (10 mL per tube). A high peak of absorbance at 280 was detected from fraction number 17 (for lactoperoxidase) and fraction number 11 (for lactoferrin) (Fig. 1). The fraction number 17-26 (for lactoperoxidase) and 11-20 (for lactoferrin) were collected and checked the protein profile using SDS-PAGE (Fig. 2). Lactoperoxidase activity from the collected eluate was analyzed resulting the value of 45 U mL^{-1}. The protein concentration of the collected eluates containing high concentration of lactoferrin was analyzed using Lowry method resulting value of 8.1 mg mL^{-1}.

Total microbe: The manufacture of Dangke consumes 3-6 h, so, these long time of treatments may sometimes have a negative effect on bacterial count of Dangke. Furthermore, the high temperature at local area may promote the growth of bacteria resulting in the upturning the elevation of bacteria. This study used phosphate buffer, lactoferrin and lactoperoxidase system for

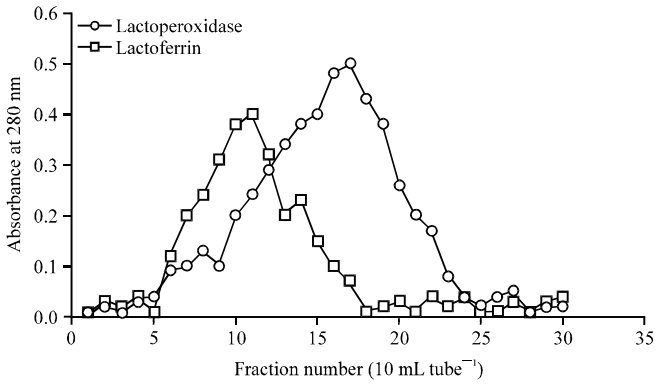

Fig. 1: Absorbance at 280 nm of the eluate from SP sepharose fast flow column (10 mL tube^{-1}) containing high concentration of lactoperoxidase and lactoferrin. The ten tube after peak was collected to analyze its protein profile using SDS PAGE

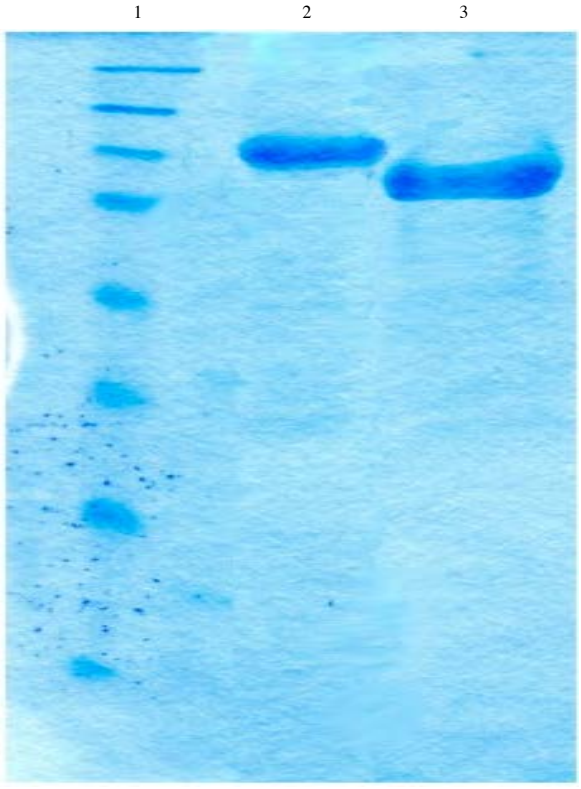

Fig. 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of eluate containing high concentration of lactoferrin, lactoperoxidase and purified from bovine milk using SP Sepharose Fast Flow. Lane 1: Standard protein from 16.5-120 kDa, Lane 2: Lactoferrin, Lane 3: Lactoperoxidase

the storage solution of Dangke. The 1 h of dipping in the storage solutions were applied then the total bacteria was calculated based on the bacteria growth in the surface area of Dangke (Fig. 3).

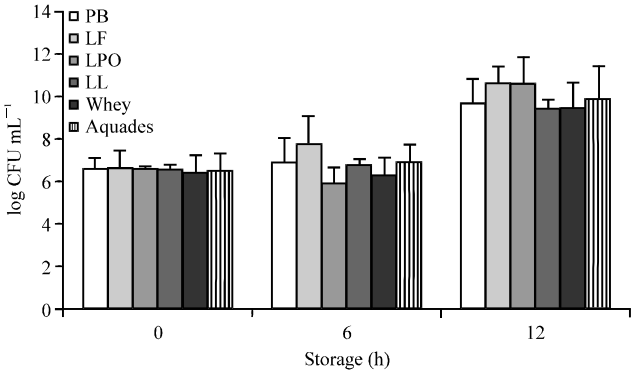

Fig. 3: Dangke total microbe with soaking treatment in solution of phosphate buffer, lactoferrin, lactoperoxidase system, lactoferrin+lactoperoxidase system, whey and pure water during the storage

Table 1: pH value of Dangke soaked in phosphate buffer, lactoferrin, lactoperoxidase system, lactoferrin+lactoperoxidase system, whey and pure water/aquades at ambient temperature

	Dangke pH value after treatment					
Storage period (h)	PB	LF	LPOS	LF+LPOS	Whey	Aquades/pure water
0	6.72±0.19	6.53±0.01	6.52±0.08	6.47±0.06	6.20±0.01	7.17±0.03
6	7.07±0.06	6.58±0.02	6.87±0.06	6.53±0.06	6.63±0.06	7.18±0.01
12	6.64±0.01	6.50±0.00	6.50±0.00	6.38±0.08	6.10±0.10	6.66±0.01
Mean	6.81^{b}	6.54^{d}	6.63^{c}	6.46^{e}	6.31^{f}	7.00^{a}

a,b,c,d,e,f value with superscript letter behind number that is different on mean line shows real difference. (x,y,z) value with superscript letter behind different number on mean column shows real difference ($p<0.05$)

Based on the figure, initial total bacteria in Dangke was detected from a range of 6.46±0.78 up to 6.64±0.80 CFU mL^{-1}. If compare to the maximum limit of total bacteria in soft cheese, i.e., 6 log CFU mL^{-1} (Indonesian National Standard, 2000), the number of total bacteria just on the limit. The amount of total bacteria on the standard limit indicating probable contamination of the milk as a result of poor hygiene and the contamination at the processing plant may increase the number of total bacteria in Dangke.

The increase of total bacteria was detected on the Dangke stored in phosphate buffer from 6.65±0.5-6.95±1.1 CFU mL^{-1}. The prolongation of incubation into 12 h resulting in the remarkable increase of total bacteria to 9.70±1.12 CFU mL^{-1}. The remarkable amount of total bacteria on Dangke stored for 12 h was detected on all treatments ranged from 9.46±0.4-10.61±0.8 CFU mL^{-1}.

The storage of Dangke in phosphate buffer, lactoferrin, lactoferrin+lactoperoxidase system, whey and pure water for 6 h slightly increased the total bacteria to the amount of total bacteria ranged from 6.36±0.7-7.70±1.3 CFU mL^{-1}. Amazingly, the lactoperoxidase system storage remarkable decreased the total bacteria from 6.59±0.1-5.95±0.7 CFU mL^{-1}.

The occurrence of the decrease of total microbe at the sixth hour using lactoperoxidase system as soaking media at ambient temperature is shown in Fig. 3. Dangke that was soaked in lactoperoxidase system had 5.95 log CFU mL^{-1} of total microbe. The result of Touch *et al.* (2004) study could reduce the amount of *S. enteretidis* in vegetable product as much as 5.4 log unit and could inhibit the organism growth for 4 h at 30°C incubation with lactoperoxidase system

treatment. Lactoperoxidase catalysed thiocyanate oxidation by hydrogen peroxide and resulted in product with antimicrobial characteristic (Seifu *et al.*, 2005) especially hypothiocyanate ion, this ion will react with membrane of bacteria cytoplasm and interrupt metabolic enzyme function and produce antimicrobial effect (Jooyandeh *et al.*, 2011). Hypothiocyanate is bacteriostatic and tends to have main part in lactoperoxidase system (Aune and Thomas, 1977).

Treatment with lactoferrin soaking at the sixth hour could not reduce total microbe, this was suggested that lactoferrin activity decreased, so that the holding capacity to iron weakened. Adlerova *et al.* (2008) reported that though lactoferrin had the ability to hold free iron, that is one of essential elements for the growth of bacteria and responsible for bacteriostatic effect. However, some bacteria can adapt with new condition and release siderophores (Iron chelat compound that is derived from bacteria) that compete with lactoferrin for Fe^{3+} ion (Crosa, 1989; Ratledge and Dover, 2000). Some types of bacteria that include in Neisseriaceae family adapt with new condition by expressing specific receptor that can hold lactoferrin and cause the change of lactoferrin molecule tertiary structure that caused iron dissociation (Ekins *et al.*, 2004).

Storage for 12 h in all treatments cannot reduce the total microbe, it was suggested that the longer the storage at ambient temperature, the higher the amount of total microbe of milk product. This is along the lines with Buckle *et al.* (1987) study stated that condition of storage temperature has effect on the amount of total microbe, it is caused by the storage temperature influences metabolism and the growth of microbe. The higher the temperature (ambient temperature 20-30°C), the faster the speed of microbe metabolism and growth, in reversed, the lower temperature (cold temperature 4°C), the slower the speed of bacteria metabolism and growth. Dangke storage in this study was stored at ambient temperature (30°C) so that the increase of the amount of total microbe on the treatment at the 12th h was occurred. The antibacterial activity of lactoperoxidase system depends on bacteria species or strain used, temperature of incubation, type of media used in activation and concentration of lactoperoxidase system components (Sarkar and Misra, 1992; Fuglsang *et al.*, 1995).

pH value: The pH value of Dangke stored in various medium at ambient temperature is presented in Table 1. It is showed that the pH of Dangke was significantly affected by medium ($p<0.05$). Dangke stored in lactoperoxidase sistem and lactoferrin were more stable in pH value if compare to other medium (the decrease were 0.3-0.4%). The less change of pH of Dangke stored in lactoperoxidase system and lactoferrin indicated less of microbial activity since the pH value may indicated the microbial activity. The remarkable decrease in pH value (1.2-7.6%) was found in Dangke stored in PB, LL, whey and aquadest. The lowest pH value was found in the whey medium since there was no buffer applied in whey. This study was used PB pH 7.0 as solvent in all applied enzymes, therefore, the minimum achieved pH of danke stored in enzymes was stable (Stoll and Blanchard, 1990). The range of pH of Dangke in all treatments were at a range 6.10±0.1-7.18±0.01, however, the sampel with enzyme treatment achieved pH at range 6.10±0.1-6.87±0.06 indicating inline the requirement of pH in milk derived product in Indonesia (from pH 6.0-7.0) (Indonesia National Standard).

Lactoperoxidase system and lactoferrin inhibited the reduction of pH value, however the combination both of these enzymes were unable to inhibit the reduction resulting in the decrease of pH from 6.47±0.06-6.38±0.08. Synergistic effect of two enzyme on antibacterial activity was found in many investigation (Murdock *et al.*, 2007; Chung and Hancock, 2000), however, as described previously LPOS and LF were unable to inhibit the decrease of pH.

CONCLUSION

It can be concluded from the result of this study that lactoperoxidase system can be used as antimicrobial agent that can reduce Dangke total microbe with 6 h incubation period at ambient temperature. The soaking using lactoferrin and lactoperoxidase system can maintain Dangke pH value.

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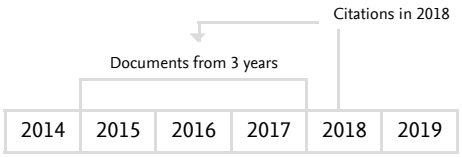

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International Journal of Dairy Science

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Scope	International Journal of Dairy Science is dedicated to disseminate the international original research on all aspect of dairy science. International Journal of Dairy Science publishes original scientific research on all aspects of dairy science including: animal husbandry, the physiology, biochemistry and endocrinology of lactation, milk production, composition, preservation, processing and separation, biotechnology and food science, properties of milk proteins and other components, dairy products such as cheese, fermented milks and spreads, relevant studies in bacteriology, enzymology and immunology, the use of milk products in other foods; and the development of methods relevant to these subjects.

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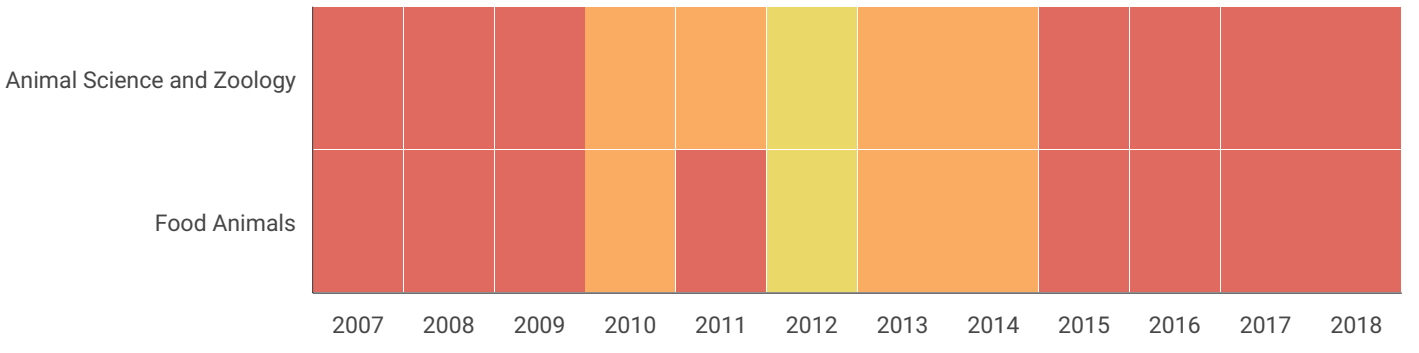

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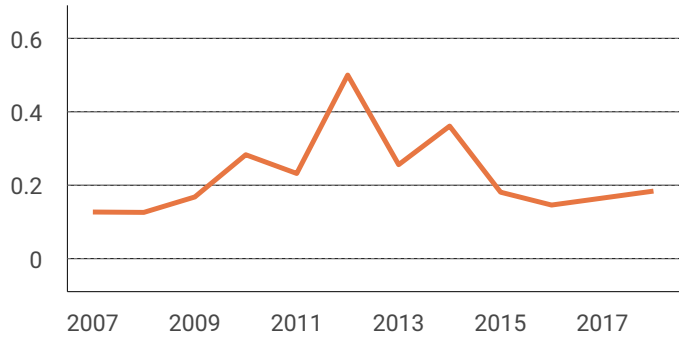

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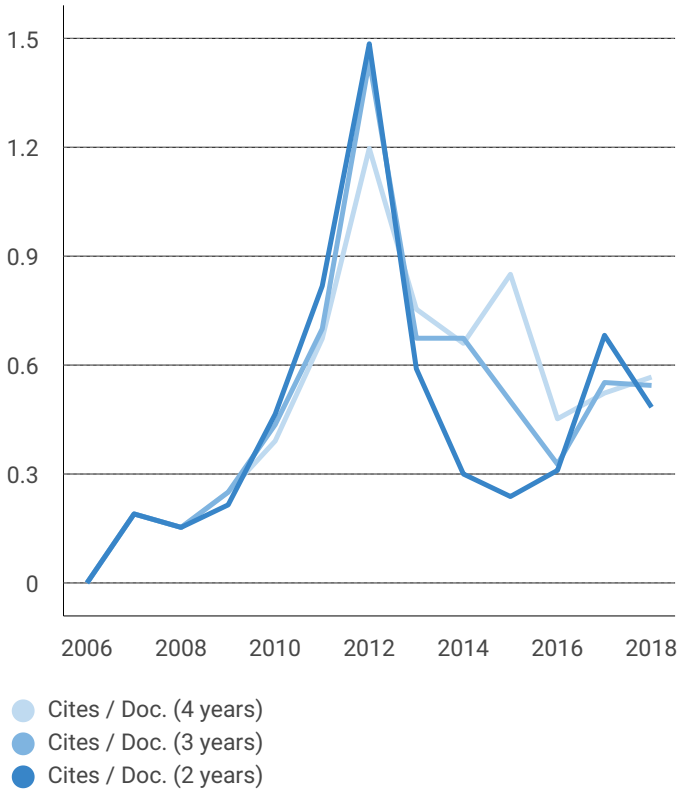

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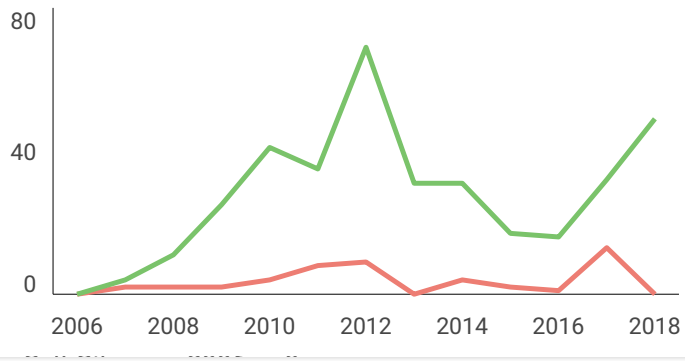

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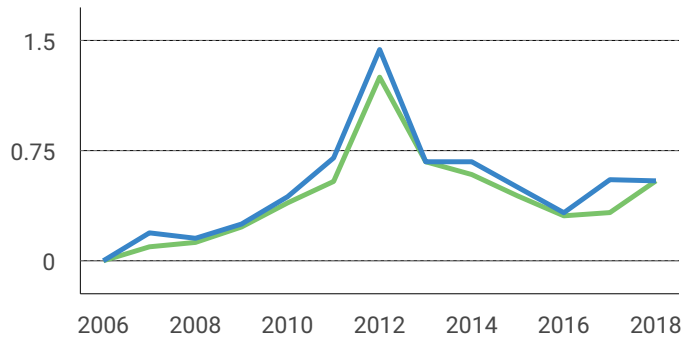

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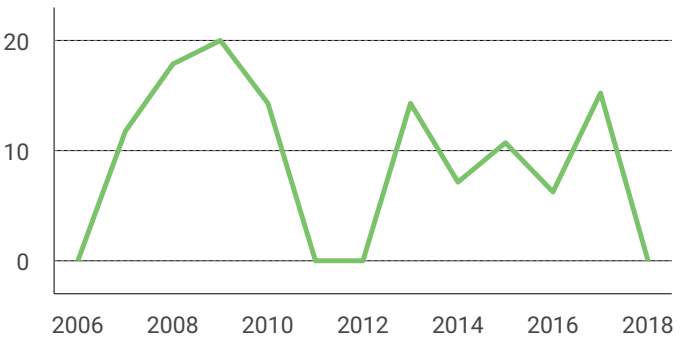

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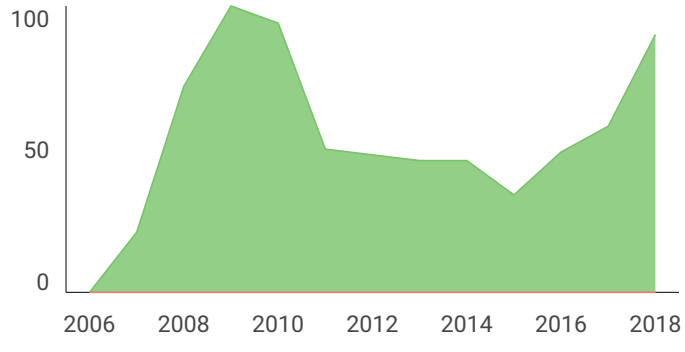

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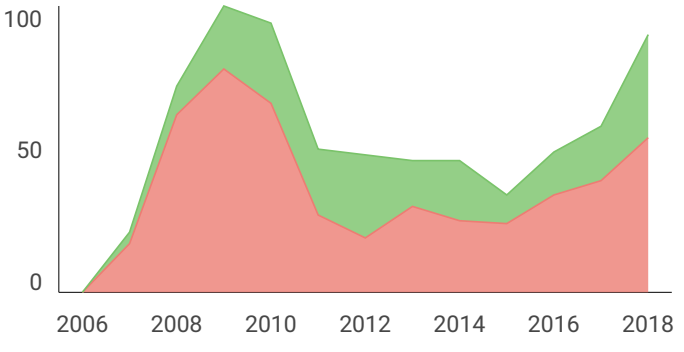

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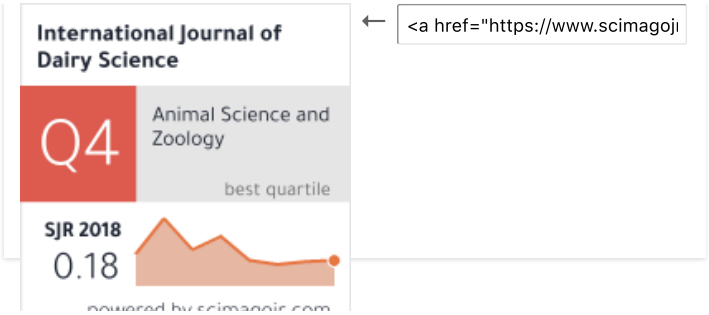
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RESISTANCE OF IMMOBILIZED LACTOPEROXIDASE ACTIVITY FROM BOVINE WHEY AGAINST STORAGE SOLUTIONS

Dwi Novrina Nawangsari[1)], Ahmad Nimatullah Al-Baarri[1)] and Sri Mulyani[2)]

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ABSTRACT

Lactoperoxidase (LPO) could be simply obtained from whey through immobilization using a cation exchange resin of SP Sepharoses. LPO received high attention since the antimicrobial properties of LPO system (LPOS) that are consisted of LPO, SCN^-, and H_2O_2 was able to generate $OSCN^-$ for strong antimicrobial agents. This study was done to analyze the immobilization efficiency of LPO onto two types of sepharose: SP-Sepharose Fast Flow (SPFF) and SP-Sepharose Big Beads (SPBB). The remaining of LPO's activity (%) against storage solution was also observed. The whey was obtained from bovine skimmed milk that was coagulated using rennet and acid lactic. The LPO was obtained from whey using SPFF. To analyze the remaining of immobilized LPO activity, the immobilized LPO was stored in pure water, phosphate buffer, milk, and whey at 10˚C. The activity of LPO was monitored for 10 days. The result indicates that the LPO could be purified from whey. The obtained LPO (35 U/ml) was attached onto SPFF and SPBB. It was concluded that 0.6 g SP-FF and 0.9 g SP-BB were able to achieved 100% of immobilization eficiency (IE). LPO activity of the immobilized LPO onto Sepharoses were able to kept until 5 days when it was stored in whey. Other storage solution remained various LPO activity during storage.

Key words: Lactoperoxidase, SP Sepharose Fast Flow, SP Sepharose Big Beads, immobilization, remaining activity.

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INTRODUCTION

Lactoperoxidase (LPO), together with SCN^- and H_2O_2 have been understood to generates intermediate product of $OSCN^-$ as antibacterial agent that has a broad spectrum of antimicrobial effects against bacteria, fungi and viruses. This antibacterial agent could be produced if these three components exists in the medium (Seifu et al., 2005, Al-Baarri, 2011). LPOS has been widely used as a preservative in dairy products and nondairy products (Seifu et al., 2004, Touch et al., 2004, FAO/WHO, 2005, Boots and Floris, 2006, Oghaiki et al, 2007, Fweja et al., 2008, Al-Baarri et al., 2011a).

It has been understood that whey contains large number of LPO therefore purification method of LPO from whey has been well developed (Touch et al., 2004, Zhou and Lim, 2009, Al-Baarri et al., 2010). SP-Sepharose has been known to provide beneficial effect for the immobilization efficiency since SP-Sepharose almost completely immobilized LPO and reusable (Al-Baarri et al., 2010). Although LPOS was widely used in food application but it still remained the problem of its expensiveness, therefore the immobilization of LPO was needed for the reuse of LPO.

SP Sepharose has been known as apropriate immobilization agent for capturing lactoperoxidase (Fee and Chand, 2006; Hayashi et al., 2012). SP Sepharose Fast Flow (SPFF) and SP Sepharose Big Beads (SPBB) were the common resin for immobilization since the they provide simply application, long term of use, and easy for reuse (Amersham-Bioscience, 2001). If compare to other immobilization agent such as chitosan, SP Sepharoses showed higher capturization of lactoperoxidase (Al-Baarri et al., 2012). In the other hand, SP Sepharose application for immobilization of LPO resulting in the much more expensive of the use of LPO, therefore the efficient use of SP Sepharose to immobilize LPO is required. Based on our knowledge, there was no documentation for the efficient use of LPO immobilization using SP Sepharose, therefore this research has been done for analyzing the maximum capturization of LPO onto SP Sepharoses. This information might provide the benefit for minimum use of SP Sepharose for LPO immobilization. Since the immobilized LPO allowed the reuse of enzyme, the appropriate storage solution for keeping the enzyme activity is needed. To answer this, this research has also been done for analyzing the remining LPO activity after storage.

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MATERIALS AND METHODS

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Materials

SP-FF and SP-BB were purchased from Amersham Pharmacia Biotech, Sweden (Lot. No.). Microbial derived rennet was purchased from Singapore. Cow's milk was obtained from Faculty of Animal and Agricultural Sciences's farm, Diponegoro University, Semarang, Indonesia. 2,20-azino-bis (3-ethylbenz-thiazoline-6-sulphonic acid) or ABTS was obtained from Kagawa Science (Lot No. 7ROZC-EC) Tokyo Chemical, Industry Co. Ltd. Japan. The spectrophotometer (Mini UV-1800, Schimadzu, Japan) was used for analysis enzyme activity. Unless other compounds specified, all other compounds were reagent grade.

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Preparation of whey

The whey was prepared as method performed by Al-Baarri et. al. (2011b) without any modification.

LPO Immobilization from Whey

The procedure for LPO imobilization was conducted as the method that was performed by Al-Baarri et al., 2010 with modifications. SP Sepharose Fast Flow (SPFF) was used as a agent for LPO immobilization from whey. Whey at the volume of 1800 ml was eluted through a glass column (3 x 40 cm) filled with 60 g of SP-FF. Prior to elution, SPFF was washed with 300 ml of phosphate buffer (PB) (pH 6,8) containing 1 M NaCl to remove unnecessary compounds. The whey was circulated through the column using feedback tubing and a peristaltic pump. The circulation was done at the flow rate of 1.0 ml/min. After draining the whey away, the resin was washed with 300 ml of 0.4 mM NaCl in 0.1 mM phosphate buffer (pH 7.0) using fraction collector (10 ml per tube) to obtain the solution containing high concentration of LPO. Three group of fractions (fraction number 1–10, 11–20, 21–30) were analyzed for protein profile using SDS PAGE to check the purity. Finally, based on the SPS PAGE analysis, the fraction number 21–30 was analyzed for LPO activity (LPO activity was 35 U/ml) and was used throughout experiment.

Determination of Captured LPO onto SP Sepharoses

SP-FF and SP-BB (0.1–1.0 g) were washed in 1 M NaCl in PB pH 7.0 and then were placed in the column (1x10 cm). The immobilization process was started with the elution of 1 ml of LPO through column. The flow rate was set into 1 ml/minute using peristaltic pump. The output was collected for measurement of remained LPO activity in the SP Sepharoses. This experiment was repeated three times and column were wash with serial elution of 1 M NaOH and pure water, respectively. The immobilized LPO was stored in milk, whey, pure

water and whey. All storage solutions were sterilized using autoclave at 110˚C for 10 minutes. Immobilized LPO was stored at 4˚C for 10 days. The remaining of LPO activity immobilized onto SP-Sepharose was calculated by eluting immobilized LPO using 1 M NaCl in PB pH 7.0.

LPO Activity Determination

LPO activity was performed as the following method: 450µl of 1.0 mM ABTS in 10 mM acetate buffer (pH 4.4) and 450 µl of 0.55 mM H_2O_2 in pure water were gently poured into the cuvette. The enzyme (50 µl) was subsequently added to cuvette. The increase of absorbance at 412 nm measured for 20 second. One unit of LPO enzymatic activity was expressed as the amount of enzyme needed to oxidize 1 µmol ABTS/min. The molar extinction coefficient of ABTS at 412nm was 32.400 M^{-1} cm^{-1} (Touch et al., 2004).

Immobilization Efficiency

The immobilization efficiency (IE) was calculated as follows: IE(%)= E1/E0 x 100, where E0 is the LPO activity added to the SP Sepharoses (U/ml) and E1 is the LPO activity embedded in the SP Sepharose (U/ml) (Al-Baarri et al., 2010).

RESULTS AND DISSCUSSION

Purification LPO

Whey has a lot of enzymes and it is available in low cost because whey is by product of dairy manufacture so it is the challenge to use whey as enzyme sources including LPO. Table 1 shows the LPO activity and band (s) of the solution obtained from the elution of 1 M NaCl in PB pH 7.0 through SPFF containing LPO. As mention in methods, SPFF containing LPO was generated from whey that was eluted through SPFF column. The result of LPO activity was 27.7±2.9, 39±4.5, and 35.2±3.4 U/ml for fraction number 1–10, 11–20, 21–30, respectively. As shown in Table 1, the highest of LPO activity was group of fraction number 11–20, however since the band of this group showed two bands indicating two protein was detected, for whole of experiment, group of fraction number 21–30 was used. This group showed single band indicating only LPO that was captured by SPFF.

In this research SPFF was used to obtain LPO since this ion exchange resin has diameter 45–165 µm resulting in the wider of surface area than SPBB Amersham-Bioscience, 2001). In line with this result, Touch et al., 2004 used SPFF for purifying LPO from whey resulting in the good ability to catch LPO (108U/ml). The activity of LPO in this research

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was less than that of other researcher since the absence of microfiltration step in this research. It has been known that the microfiltration might concentrate the enzyme resulting in the high activity of LPO.

Immobilization Efficiency

Immobilization efficiency plays an important role for determination of immobilization agent. This research determined immobilization efficiency (IE) of LPO using SPFF dan SPBB (Figure 1). The volume of SP Sepharoses used in this experiment had a range from 0.1 to 1.0 g to catch the LPO at initial activity of 35.2±3.4 U/ml. The increase of IE was found as an increase of SP Sepharoses's weight. When 0.6 g of SPFF was employed, the IE achieved 100% indicating all of LPO employed was able to be captured by SPFF. When the weight of SPFF was increased, the IE was in steady state maximumly.

An increase SPBB from 0.1 to 1.0 g elevated the IE from 38.6 to 100%. However, 0.9 g of SPBB completely captured LPO resulting the IE of 100%. One gram of SPFF was reported to have a maximum capture of LPO in 300 ml whey (equal to 750 U/ml LPO activity) (Al-Baarri et al., 2010). This can be explained that the capture might be depend on the quantity of enzymes per mililiter. This research used high activity of LPO resulting in the loss of LPO activity.

Since the SPFF and SPBB provided the maximum IE at 0.6 and 0.9 g, respectively, thus these amount of SP Speharoses has been used in the rest of experiment. The LPO immobilized onto SPFF and SPBB was stored in the various storage solutions: pure water, PB, milk, and whey for 10 days in 10˚C.

Remaining LPO Activity During Storage

The percentage of remaining immobilized LPO activity stored at 10°C for 10 days in various storage solutions is shown in Figure 3. LPO activity of immobilized LPO was measured after purging the LPO attached onto SP Sepharose with 1 M NaCl in PB pH 7.0. The percentage of remaining LPO activity was determined by comparing the LPO activity after storage to the initial immobilized LPO at first day of storage.

The percentage of LPO activity attached onto SPBB and SPFF is shown on Figure 3a and 3b, respectively. Based on Figure 3a, whey was able to maintain 100% LPO activity within 4 days. The extention of storage time resulted in the remarkable reduction of remaining LPO activity. Milk was able to maintain the LPO activity at seven days of storage even though the remaining of LPO activity at that time was very negligible in amount (2.3%).

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As previously mentioned, whey was able to completely keep LPO activity within 4 days of storage. This can be explained that whey components support the activity of LPO. It has been studied that LPO activity might be inhibited by casein (Singh et al., 2009) while the casein has been removed from whey.

The remaining of LPO activity attached onto SPFF during 10 days of storage at 10˚C is shown on Figure 3b. PB was able to maintain 100% of LPO activity within 5 days of storage. The milk and whey were able to keep 100% of LPO activity within 3 days of storage. Based on the availability, since LPO was derived from whey, the storage of immobilized LPO in whey should keep its activity up to 5 days of storage. Therefore it is suggested that immobilized LPO should be stored in whey.

CONCLUSION

The results can be concluded that LPO could be purified from whey using fraction number 21–30. One mililiter of LPO (35 U/ml) could be completely immobilized onto 0.6 g SPFF or 0.9 g SPBB (immobilization efficiency was 100%). Among various storage solution, whey was able to keep 100% of LPO activity up to 5 days of storage.

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Table 1. LPO activities of solution obtained from dilution of 1 M of NaCl in PB pH 7.0 through captured LPO from bovine whey onto SP Sepharose Fast Flow in three groups of fraction number. Data were obtained from three replications.

	Group of fraction number		
	1–10	11–20	21–30
LPO Activity (U/ml)	27.7±2.9	39.5±4.5	35.2±3.4
Band (s)	Double bands	Double bands	Single band

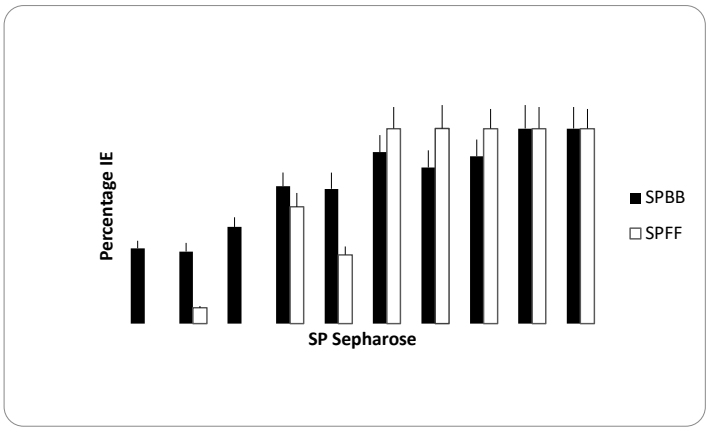

Figure 1. Immobilization efficiency of SPBB and SPFF using various weight.

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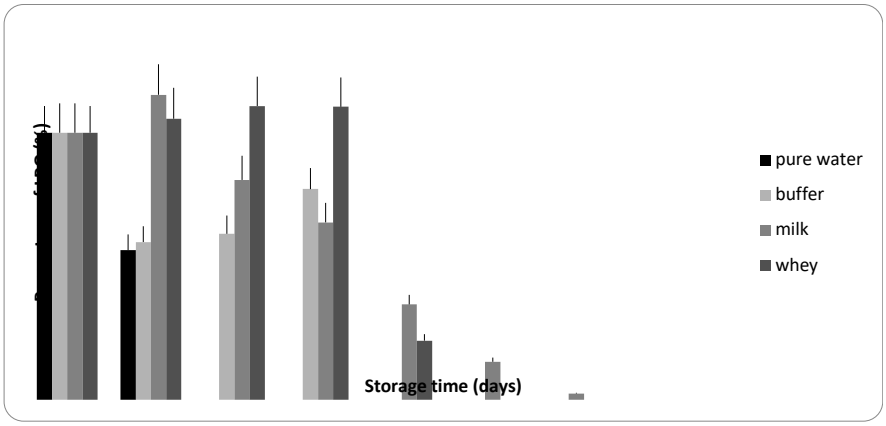

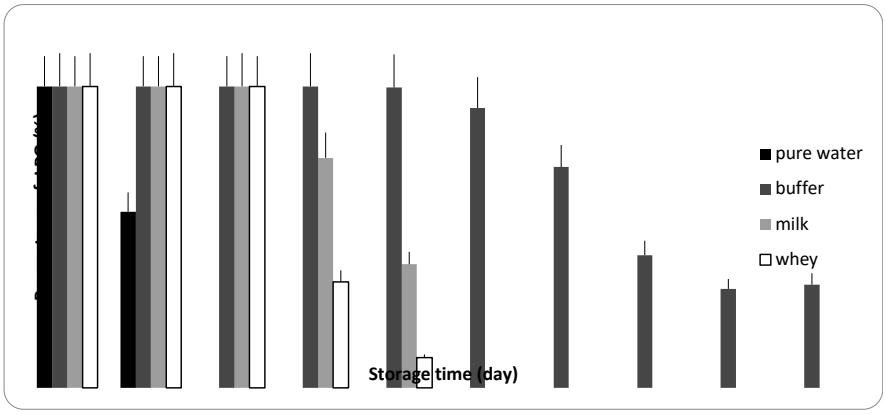

Figure 2 The percentage of LPO activity attached onto SPBB and SPFF during ten days of storage in various storage solution.

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ABSTRACT: • Abstract section need some modifications; at the start describe the aim and background of study in a couple of sentences. In 2-3 sentences give a general sketch of methodology and then explain significant results of study. If required then use numeric values in support of your significant results. End this paragraph with a concluding statement and its length should not exceed from 250 words. Avoid long and confusing sentences on average write 27±5 words per sentence. In case abbreviations are used then define them properly, an abbreviation should be defined at the place where it was used for the very first time both in abstract and rest of the article, separately.	√	
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Dear IJDS Editor
The table below is the correction list for the article entitled:
TOTAL BACTERIA AND pH OF DANGKE PRESERVED USING NATURAL ANTIMICROBIAL LACTOFERRIN AND LACTOPEROXIDASE FROM BOVINE WHEY

It is our hope that you are able to accommodate this revision.

It was written	**It should be**
"Nimatullah" (page 1 in the author's name)	"Ni'matullah"
Corresponding author: Rasbawati, Department of Animal Science, Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang, 50275, Indonesia	Corresponding author: Ahmad Ni'matullah Al-Baarri, Department of Food Technology, Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang, 50275, Indonesia
"...more concern on their health." (page 2 line 10)	"...more concern on their health, the chemicals-based-preservation may be avoided."
"Table 1" (page 5, last paragraph)	"Figure 3"
"...at the 12th h occured." (page 6, line 23)	"...at the 12th h was occured."
"... and lactoferrin was inhibited the reduction of pH ..." (page 6, last paragraph)	"... and lactoferrin inhibited the reduction of pH ..."
	Please allow us to add acknowledgment. ACKNOWLEDGEMENT CONTENT: The corresponding author is highly indebted to the Ministry of National Education of Indonesia Republic for providing financial support for this research. Authors are also thankful to Prof. S. Hayakawa and Prof. M. Ogawa (Kagawa University, Japan) for their support for SDS PAGE analysis.

Sincerely,
Ahmad Ni'matullah Al-Baarri
Coresponding author

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Total Bacteria and pH of Dangke Preserved Using Natural Antimicrobial Lactoferrin and Lactoperoxidase from Bovine Whey

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ABSTRACT

Dangke is the Indonesian cheese produced from bovine milk using latex from young papaya to coagulate casein. Dangke is generally consumed by Indonesian people located in South Sulawesi Province. In line with demand of Dangke, the preservation is needed. Since, there was no literature which was found about preservation of Dangke, this study is aimed at knowing the quality of Dangke based on total bacteria and pH value stored in antimicrobial agent of lactoferrin and lactoperoxidase system from bovine whey, aquadest and phospate buffer at ambient temperature for 12 h. The lactoferrin, lactoperoxidase and whey were obtained from bovine milk and purified using ion exchange chromatography method. The result of the study showed that lactoperoxidase system provide remarkable effect of decreasing total bacteria from 8 log CFU mL^{-1} to 5 log CFU mL^{-1} while other storage solutions have no antimicrobial activity against bacteria in Dangke. The pH value of Dangke was stable when stored in lactoferrin and lactoperoxidase system. Since, both of these preservatives could be categorized as safe, the application in Dangke may open the alternative method to store Dangke.

Key words: Dangke, lactoferrin, lactoperoxidase, whey, total bacteria

INTRODUCTION

Dangke is a traditional cheese from South Sulawesi Province in Indonesia. Dangke is mostly made from cow's milk but buffalo's milk or their mixture can also be used. Dangke is a semi solid and salty cheese that available in the traditional market and traditionally manufactured by local people. A small amount of papain has been used to coagulate casein from whey. After whey removal, the mild pressure is usually applied to produce semi solid cheese. The compositions of Dangke are 47.75% of water, 2.32% of ash, 33.89% of fat and 17.01% protein (Marzoeki *et al.*, 1978). The process of making Dangke initially is started by heating in low temperature for long time (65°C, 30 min) and for casein coagulation, subsequently 5 g of papain is added into milk. The addition of papain exerts bitter taste since the papain may promote the hydrophobic groups generation (Amri and Mamboya, 2012). The bitterness taste of Dangke may be neutralized by the addition of salt. It has been understood that salt may also inhibit the spoilage of bacteria (Beresford *et al.*, 2001). Native people commonly consume Dangke for the complimentary of their food, so the salt may promote the better taste in food (Sirajuddin *et al.*, 2013).

Dangke manufacturing is mostly made from cow's milk but sheep's and goat's milk or a mixture of them. Since, the local people consume Dangke daily, they did not pay high attention for the preservation because local people will consume it immediately after manufacturing. However, since the number of local people is travelling from and to this province, the demand has increase resulting in the need for preservation. Natively, Dangke's shelf live is relatively short (about six hours), this is because Dangke is made from fresh milk that contains various elements and mostly consists of food substance that is also needed for bacteria growth. One of methods to extend the storage period of food product is the preservation by using antimicrobial substances or compounds.

The preservative for prolonging the shelf live of Dangke may be obtained from chemicals however, since the people may pay much more concern on their health, in line with this demand, researchers pay much more attention for the utilization of the Generally Recognize As Safe (GRAS)'s preservatives. Lactoferrin or most commonly called lactotransferrin is transferrin that is isolated from milk. Lactoferrin is antimicrobial agent because it contains glycoprotein-703 amino acid that has extremely high ability to bind Fe from microbe, so that it significantly inhibits microbe growth (Conneely, 2001). Lactoperoxidase system is widely known as a system that naturally exists in fresh milk as antimicrobial. Lactoperoxidase system has been proven for being active to positive and negative gram microorganism (Naidu, 2000; Marks *et al.*, 2001). Lactoperoxidase system catalyses reaction of hydrogen peroxide (H_2O_2) and thiocyanate (SCN^-) that occur naturally in milk to become a compound named hypothiocyanite ($OSCN^-$) (Barrett *et al.*, 1999; Kussendrager and van Hooijdonk, 2000; Seifu *et al.*, 2007). The $OSCN^-$ is a compound that takes responsibility for killing bacteria, fungi and virus by breaking down sulfhydryls groups (S-H group) from cell membrane causing vital impairment of cell membrane finally leading to the death of the cell (Al-Baarri *et al.*, 2011a; Borch *et al.*, 1989; Dajanta *et al.*, 2008; Touch *et al.*, 2004).

Based on the remarkable antimicrobial activity of lactoferrin and lactoperoxidase system and there is no study that was found in the preservation of both compound in Dangke, this study was aimed at analysis of total bacterial growth and pH value of Dangke stored at ambient temperature. The result of this study may provide an alternative way for Dangke's storage.

MATERIAL AND METHODS

Materials: Fresh bovine milk was provided by Campus Farm in Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang-Indonesia. Papain enzyme was obtained from 3-4 month old fresh papaya fruits. Commercial microbial rennet was obtained from Singapore. The spectrophotometer (Mini UV-1800, Schimadzu, Japan) was used for analysis of LPO activity and detection of protein concentration. The H_2O_2, KSCN, 2, 2-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid) (ABTS) were purchased from Sigma. Unless other specified compound were reagent grade.

Whey preparation: The whey was prepared as method conducted by Al-Baarri *et al.* (2011b) without any modification.

LPO production from whey: Whey was used for production of lactoperoxidase and lactoferrin through ion exchange method using SP Sepharose Fast Flow Column (GE Healthcare Bio-Science AB, Sweeden, Lot. No. 10081054). Subsequently, 0.4 M NaCl in 300 mL of 0.1 M PB (pH 7.0) was flowed into SP Sepharose® Fast Flow in order to generate lactoperoxidase solution. Three hundred millilitres of 1 M NaCl in 0.1 M PB (pH 7.0) was then poured to produce lactoferrin solution. Each

eluate obtained from above mentioned method was analyzed for approximate protein concentration in each tubes (10 mL tube^{-1}) using spectrophotometer and its absorbance was measured at 280 nm. Top ten highest absorbance of tubes after peak were collected to determine the LPO enzyme activity using ABTS at 412 nm (Al-Baarri *et al.*, 2011b). To check the purity of lactoperoxidase and lactoferrin, the SDS PAGE was applied.

Manufacture of Dangke: Procedure of Dangke's making was adapted from method of JICA (2009). It was started by a heating of 3 L of fresh bovine milk at 60°C for 30 min. The next step was the addition of 0.03% (v/v) papain enzyme. After aglutination occurred, the whey was drained by using sterile filter cloth. The curd was then stored in ambient temperature and gently pressed for 3 h to produce the Dangke.

Microbial count: Petrifilm Aerobic Count Plates (3 M Microbiology, St. Paul, Minn., U.S.A.) was used to count the microbial appeared in Dangke. After manufacture, Dangke was cut into cube with the approx. of weight 1 g. The number of total bacteria in Dangke in the presence of lactoperoxidase system was determined as follows: 1 g Dangke was stored at 1000 µL hyphothiocyanite-rich-solution and incubated for 6 h at 30°C. Hyphothiocyanate-rich-solution was made from the addition of 250 µL of 1.0 mM H_2O_2 and 250 µL of 1.0 mM KSCN into 500 µL of LPO solution (35 U mL^{-1}). After incubation at 30°C for 10 min, hyphothiocyanite-rich-solution should be generated. Enumeration of bacteria was done by counting the solution that was obtained from serial dilutions of the assay mixture with a sterile 0.88% NaCl solution. The diluted mixture (1000 µL) was spread onto plates. The plate were incubated at 37°C for 48 h. The CFU of microbes in the sample solution were counted on the plates.

Statistical analysis: Total bacteria of Dangke stored in various storage solutions for 12 h were analyzed statistically using one-way analysis of variance (ANOVA) and the means were compared by the Duncan test at a significant level of 0.05 (Free Statistical Software Package R for Macintosh, U.S.A).

RESULT AND DISCUSSION

Purification of lactoperoxide and lactoferrin: Lactoperoxidase and lactoferrin was obtained from whey using ion exchange chromatography method. Both components were collected from top ten highest absorbance of tubes after peak at 280 nm (10 mL per tube). A high peak of absorbance at 280 was detected from fraction number 17 (for lactoperoxidase) and fraction number 11 (for lactoferrin) (Fig. 1). The fraction number 17-26 (for lactoperoxidase) and 11-20 (for lactoferrin) were collected and checked the protein profile using SDS-PAGE (Fig. 2). Lactoperoxidase activity from the collected eluate was analyzed resulting the value of 45 U mL^{-1}. The protein concentration of the collected eluates containing high concentration of lactoferrin was analyzed using Lowry method resulting value of 8.1 mg mL^{-1}.

Total microbe: The manufacture of Dangke consumes 3-6 h, so, these long time of treatments may sometimes have a negative effect on bacterial count of Dangke. Furthermore, the high temperature at local area may promote the growth of bacteria resulting in the upturning the elevation of bacteria. This study used phosphate buffer, lactoferrin and lactoperoxidase system for

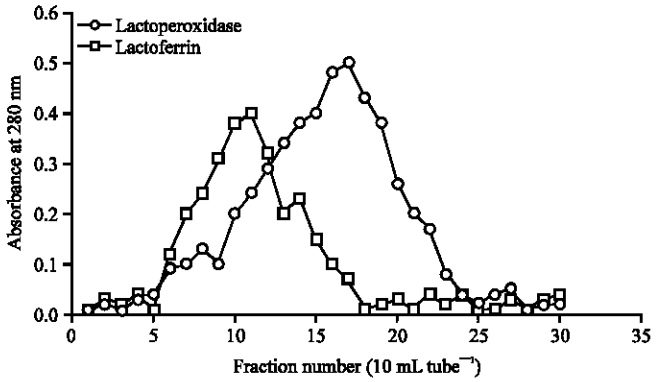

Fig. 1: Absorbance at 280 nm of the eluate from SP sepharose fast flow column (10 mL tube^{-1}) containing high concentration of lactoperoxidase and lactoferrin. The ten tube after peak was collected to analyze its protein profile using SDS PAGE

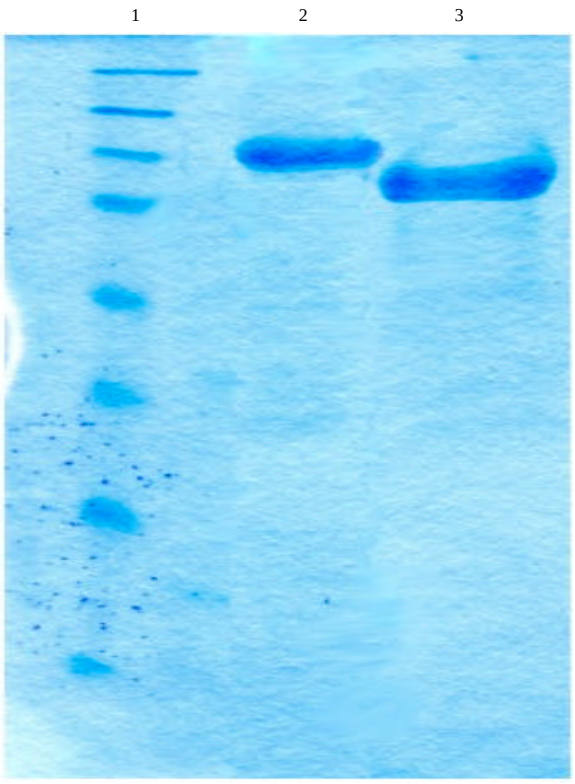

Fig. 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of eluate containing high concentration of lactoferrin, lactoperoxidase and purified from bovine milk using SP Sepharose Fast Flow. Lane 1: Standard protein from 16.5-120 kDa, Lane 2: Lactoferrin, Lane 3: Lactoperoxidase

the storage solution of Dangke. The 1 h of dipping in the storage solutions were applied then the total bacteria was calculated based on the bacteria growth in the surface area of Dangke (Fig. 3).

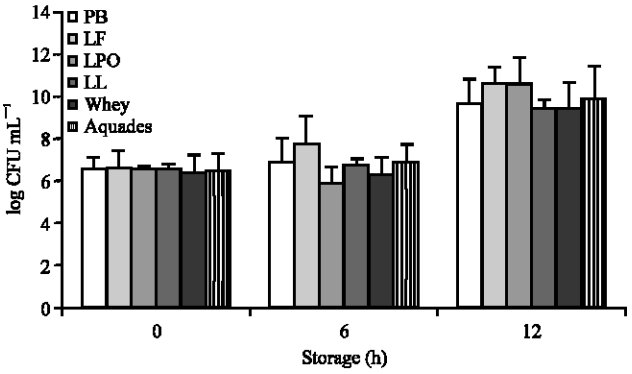

Fig. 3: Dangke total microbe with soaking treatment in solution of phosphate buffer, lactoferrin, lactoperoxidase system, lactoferrin+lactoperoxidase system, whey and pure water during the storage

Table 1: pH value of Dangke soaked in phosphate buffer, lactoferrin, lactoperoxidase system, lactoferrin+lactoperoxidase system, whey and pure water/aquades at ambient temperature

	Dangke pH value after treatment					
Storage period (h)	PB	LF	LPOS	LF+LPOS	Whey	Aquades/pure water
0	6.72±0.19	6.53±0.01	6.52±0.08	6.47±0.06	6.20±0.01	7.17±0.03
6	7.07±0.06	6.58±0.02	6.87±0.06	6.53±0.06	6.63±0.06	7.18±0.01
12	6.64±0.01	6.50±0.00	6.50±0.00	6.38±0.08	6.10±0.10	6.66±0.01
Mean	6.81^{b}	6.54^{d}	6.63^{c}	6.46^{e}	6.31^{f}	7.00^{a}

a,b,c,d,e,f value with superscript letter behind number that is different on mean line shows real difference. (x,y,z) value with superscript letter behind different number on mean column shows real difference ($p<0.05$)

Based on the figure, initial total bacteria in Dangke was detected from a range of 6.46±0.78 up to 6.64±0.80 CFU mL^{-1}. If compare to the maximum limit of total bacteria in soft cheese, i.e., 6 log CFU mL^{-1} (Indonesian National Standard, 2000), the number of total bacteria just on the limit. The amount of total bacteria on the standard limit indicating probable contamination of the milk as a result of poor hygiene and the contamination at the processing plant may increase the number of total bacteria in Dangke.

The increase of total bacteria was detected on the Dangke stored in phosphate buffer from 6.65±0.5-6.95±1.1 CFU mL^{-1}. The prolongation of incubation into 12 h resulting in the remarkable increase of total bacteria to 9.70±1.12 CFU mL^{-1}. The remarkable amount of total bacteria on Dangke stored for 12 h was detected on all treatments ranged from 9.46±0.4-10.61±0.8 CFU mL^{-1}.

The storage of Dangke in phosphate buffer, lactoferrin, lactoferrin+lactoperoxidase system, whey and pure water for 6 h slightly increased the total bacteria to the amount of total bacteria ranged from 6.36±0.7-7.70±1.3 CFU mL^{-1}. Amazingly, the lactoperoxidase system storage remarkable decreased the total bacteria from 6.59±0.1-5.95±0.7 CFU mL^{-1}.

The occurrence of the decrease of total microbe at the sixth hour using lactoperoxidase system as soaking media at ambient temperature is shown in Table 1. Dangke that was soaked in lactoperoxidase system had 5.95 log CFU mL^{-1} of total microbe. The result of Touch *et al.* (2004) study could reduce the amount of *S. enteretidis* in vegetable product as much as 5.4 log unit and could inhibit the organism growth for 4 h at 30°C incubation with lactoperoxidase system

treatment. Lactoperoxidase catalysed thiocyanate oxidation by hydrogen peroxide and resulted in product with antimicrobial characteristic (Seifu *et al.*, 2005) especially hypothiocyanate ion, this ion will react with membrane of bacteria cytoplasm and interrupt metabolic enzyme function and produce antimicrobial effect (Jooyandeh *et al.*, 2011). Hypothiocyanate is bacteriostatic and tends to have main part in lactoperoxidase system (Aune and Thomas, 1977).

Treatment with lactoferrin soaking at the sixth hour could not reduce total microbe, this was suggested that lactoferrin activity decreased, so that the holding capacity to iron weakened. Adlerova *et al.* (2008) reported that though lactoferrin had the ability to hold free iron, that is one of essential elements for the growth of bacteria and responsible for bacteriostatic effect. However, some bacteria can adapt with new condition and release siderophores (Iron chelat compound that is derived from bacteria) that compete with lactoferrin for Fe^{3+}ion (Crosa, 1989; Ratledge and Dover, 2000). Some types of bacteria that include in Neisseriaceae family adapt with new condition by expressing specific receptor that can hold lactoferrin and cause the change of lactoferrin molecule tertiary structure that caused iron dissociation (Ekins *et al.*, 2004).

Storage for 12 h in all treatments cannot reduce the total microbe, it was suggested that the longer the storage at ambient temperature, the higher the amount of total microbe of milk product. This is along the lines with Buckle *et al.* (1987) study stated that condition of storage temperature has effect on the amount of total microbe, it is caused by the storage temperature influences metabolism and the growth of microbe. The higher the temperature (ambient temperature 20-30°C), the faster the speed of microbe metabolism and growth, in reversed, the lower temperature (cold temperature 4°C), the slower the speed of bacteria metabolism and growth. Dangke storage in this study was stored at ambient temperature (30°C) so that the increase of the amount of total microbe on the treatment at the 12th occurred. The antibacterial activity of lactoperoxidase system depends on bacteria species or strain used, temperature of incubation, type of media used in activation and concentration of lactoperoxidase system components (Sarkar and Misra, 1992; Fuglsang *et al.*, 1995).

pH value: The pH value of Dangke stored in various medium at ambient temperature is presented in Table 1. It is showed that the pH of Dangke was significantly affected by medium ($p<0.05$). Dangke stored in lactoperoxidase sistem and lactoferrin were more stable in pH value if compare to other medium (the decrease were 0.3-0.4%). The less change of pH of Dangke stored in lactoperoxidase system and lactoferrin indicated less of microbial activity since the pH value may indicated the microbial activity. The remarkable decrease in pH value (1.2-7.6%) was found in Dangke stored in PB, LL, whey and aquadest. The lowest pH value was found in the whey medium since there was no buffer applied in whey. This study was used PB pH 7.0 as solvent in all applied enzymes, therefore, the minimum achieved pH of danke stored in enzymes was stable (Stoll and Blanchard, 1990). The range of pH of Dangke in all treatments were at a range 6.10±0.1-7.18±0.01, however, the sampel with enzyme treatment achieved pH at range 6.10±0.1-6.87±0.06 indicating inline the requirement of pH in milk derived product in Indonesia (from pH 6.0-7.0) (Indonesia National Standard).

Lactoperoxidase system and lactoferrin inhibited the reduction of pH value, however the combination both of these enzymes were unable to inhibit the reduction resulting in the decrease of pH from 6.47±0.06-6.38±0.08. Synergistic effect of two enzyme on antibacterial activity was found in many investigation (Murdock *et al.*, 2007; Chung and Hancock, 2000), however, as described previously LPOS and LF were unable to inhibit the decrease of pH.

CONCLUSION

It can be concluded from the result of this study that lactoperoxidase system can be used as antimicrobial agent that can reduce Dangke total microbe with 6 h incubation period at ambient temperature. The soaking using lactoferrin and lactoperoxidase system can maintain Dangke pH value.

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